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Extraction and Characterization of PHB from *Acidiphilium cryptum* DX1-1

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Abstract: A new bacterial strain, was designated as strain *Acidiphilium cryptum* DX1-1, accumulates intracellular poly-β-hydroxybutyrate particles, four methods which have advantages and disadvantages for each were employed to extract PHB. Chloroform-sodium hypochlorite method is the best in extracting PHB form *Acidiphilium cryptum* DX1-1. The extraction rate reaches 73%, the purification rate is 92% and molecular weight is 326 kg/mol. Then the PHB extracted by this method was analyzed by ultraviolet-visible absorption spectroscopy, fourier transform infrared (FT-IR) and nuclear magnetic resonance (NMR). The results show that PHB from strain DX1-1 has the same biochemical structure and character with PHB standard. Mass spectrometer (MS) analysis reveals that the long chain of PHB is destroyed when treated by chloroform-sodium hypochlorite. The differential scanning calorimetry (DSC) of PHB shows PHB from *Acidiphilium cryptum* DX1-1 has low degree of crystallinity which makes the PHB has a wider range of applications.

Key words: *Acidiphilium cryptum*; PHB; extraction; characterization

1 Introduction

Petrochemical-based plastics have become one of the most applied materials because of their versatility, outstanding technical properties and relatively low cost. The accumulation of plastic wastes has become a major concern in terms of the environment[1]. Producing plastics from materials that can be readily eliminated from our biosphere in an “environmentally friendly” fashion has attracted special interest[2]. Bioplastics are natural biopolymers that are synthesized and catabolized by various organisms[3, 4], and these materials do not cause toxic effects in the host and have certain advantages over petroleum-derived plastic[5]. Poly-β-hydroxybutyrate (PHB), a commonly used bioplastic, is a kind of polyester and homopolymer widely produced and naturally accumulated by a large number of bacteria as intracellular granules in response to unfavorable growth conditions and nutrient imbalance[6-8]. Current applications of PHB-based polymers or composites include the packaging industry, medicine, pharmacy, agriculture, food industry, raw material for enantiomerically pure chemicals and the paint industry[9]. Under appropriate growth conditions, PHB can be accumulated, for examples, up to 85% of the cellular dry weight of *Alcaligenes eutrophus*[6] and 70%-90% in recombinant *Escherichia coli* cells[10, 11]. However, the use of PHB produced by bacterial fermentation as a commodity polymer is limited by its high production cost[12]. In the large-scale PHB production, the cost effectiveness and pharmacological purity of PHB are mainly dependent on the organism and the extraction method. Majority of the separation processes use solvents such as methylene chloride, propylene carbonate, dichloroethane or chloroform to extract PHB[9]. The method in which boiling chloroform is used in a Soxhlet apparatus[13, 14] is most popular. Comparing with other PHB producing organisms, the gram-negative strain *Acidiphilium cryptum* DX1-1 has low requests for nutrition and conditions of fermentation. It grows aerobic at temperatures ranging from 20 ℃ to 35 ℃ and pH ranging from 2.0 to 5.0. Strain DX1-1 is characterized as a facultatively autotrophic bacterium. It utilizes various carbon sources such as glucose，maltose，glycerin and

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starch to accumulate PHB and PHB accounts for 89% of the total cell dry mass[15]. *Acidiphilium cryptum* DX1-1 oxidizes sulphur to get energy without PHB accumulating. However, with addition of 0.1% glucose to the culture medium (sulphur is employed as the only energy), transmission electron microscope observations of negatively strain DX1-1 revealed the presence of PHB particles which take up 85% of dry cell weight. CO_2 in air can be utilized as the carbon source of PHB accumulation. So the PHB production based on *Acidiphilium cryptum* DX1-1 can reduce the cost of feed materials and production cost, and it has great potential for application in industry. This paper investigated the methods of extraction PHB from *Acidiphilium cryptum* DX1-1 and characterized the intracellular polymers PHB.

2 Experimental

2.1 Strain

Ac. cryptum DX1-1 (DQ529311) used was isolated from AMD taken from a reservoir in Dexing mine, Jiangxi Province of China and conserved by our laboratory.

2.2 Medium and culture conditions

The strain DX1-1 was maintained in medium containing the following reagents (per liter): $(NH_4)_2SO_4$ 3 g, KCl 0.1 g, K_2HPO_4 0.5 g, $MgSO_4·7H_2O$ 0.5 g, $Ca(NO_3)_2$ 0.01 g, glucose 10 g, and the medium was adjusted to pH 3.0 with H_2SO_4, and cultured at 30 ℃.

2.3 Extraction of intracellular polymer PHB[16, 17]

The cells were harvested by centrifugation at 10000 r/min for 10 min. The following methods were employed to extract PHB.

Dodecyl sulfonic acid sodium salt-sodium hypochlorite (SDS-NaClO): 3.5 mL SDS(100 g/L) was added to 0.6 g cells which was suspended in 30 mL distilled water. The mixture was incubated at 60 ℃ for 10 min, then 1.6 mL of NaClO (pH 10) solution was added and incubated at room temperature for 5 min. After centrifuged at 6000 for 20 min, the precipitate was washed with distilled water and ethanol. 15 mL of chloroform was added and the mixture was incubated at 60 ℃ for 60 min. After centrifuged, the chloroform solution containing the PHB was transferred and chloroform was removed by vacuum rotary evaporation. The PHB was washed with ethanol then dried at 60 ℃.

Lysozyme: 37.5 mL of Tris-HCl buffer (50 mmol/L, pH 7. 3) was added to 0.6 g of cells suspended in 7.5 mL of phosphate buffer solution (pH 7.4). 0.5 mL of cell lysozyme was added to the mixture, then incubated at 37.5 ℃ for 60 min. After centrifuged at 6000 r/min for 20 min, the precipitate was washed and treated by chloroform as above.

Ultrasonication: 0.6 g of cells were suspended in 30 mL of distilled water. The mixture was treated in ultrasonic crusher (set to: broken time 2 s, space time 3 to 4 s, for 20 times) for 5 min. Repeated 3 times with 1 min of interval. After centrifuged at 6000 r/min for 20 min, the precipitate was washed and r/min ted by chloroform as above.

Chloroform–hypochlorite: 15 mL of chloroform-sodium hypochlorite (pH10, *v/v*: 1:1) was added to 0.6 g of cells, then the mixture was incubated at 30 ℃ for 90 min. After centrifuged at 8000 rpm for 15 min, the supernatant was transferred into the 15 mL cold ethanol (0-4 ℃) and kept at 0 ℃ for 30 min. After centrifuged, the mixture reveals three layers and the super layer was chloroform solution with PHB. The chloroform solution was separated and treated as above.

2.4 Characterization of PHB

2.4.1 UV-visible absorption spectroscopy

The spectrophotometry was performed by using a spectrophotometer specord S 100, Analytic Jena, Germany. The sample containing the polymer in chloroform was transferred to a clean test tube. The chloroform was evaporated and 10 mL of concentrated H_2SO_4 was added. The solution was heated in a water bath for 20 min. After cooling and thorough mixing the absorbance of the solution was measured at 235 nm against H_2SO_4 blank. To further confirm the presence of PHB, absorption spectra (190-1000 nm) of the sample as well as the standard (DL-hydroxybutyric acid, Sigma Chemical Co., USA) were taken in the specord S 100 spectrophotometer following acid digestion.

2.4.2 Fourier transform infrared (FT-IR) spectroscopy

For FTIR analyses, the samples were first dissolved in chloroform and then added to KBr pellets. After complete solvent evaporation, FTIR spectra were recorded using a spectrometer Nexus 670, Nicolet, USA. A total of 20 scans were recorded at a 2 cm^{-1} resolution, between 4000 and 400 cm^{-1}.

2.4.3 Mass spectrometry (MS) analysis

2 mL of acidified methanol (containing sulfuric acid 3% *v/v*) and 2 mL of chloroform were added to 20 mg PHB. The mixture was heated at 100 ℃ for 4 h, then was analyzed by MS.

2.4.4 Nuclear magnetic resonance (NMR) analysis

$CDCl_3$ was used as the solvent. 5 mm bb probe (width at 26507.6 H z) was used to observer and record Carbon NMR (^{13}C-NM R)at 125.677 MHz; 5 mm PFG probe (width at 6068.9 H z) was used to observe and record hydrogen NMR (1H - NM R) at 499.807 MHz.

2.4.5 Differential scanning calorimetry (DSC)

The thermal properties of the samples were determined from 20 ℃ to 560 ℃ by a thermal analyzer, Netzsch STA 449C, Germany. Approximately 5 mg of sample was used for analysis. From the heating runs melting temperature (T_m) was obtained. The degree of crystallinity (X_C) was determined from the ratio of the melting enthalpy of the sample (ΔH_m) to the melting enthalpy ($\Delta H^0{}_m$) of 100% crystalline PHB (Inoue and Yoshie, 1992).

3 Results and Discussion

3.1 *Ac. cryptum* DX1-1 and intracellular PHB

After 2 days of incubation on agar plates at 30 ℃, the bacterial isolate forms milky, translucent, convex, and entirely smooth colonies. The cells are short rods (1.9±0.1 μm)×(0.7±0.05 μm) observed under scan electron microscope, and PHB granules accumulate inside the cells observed by transmission electron microscopy as shown in Fig.1.

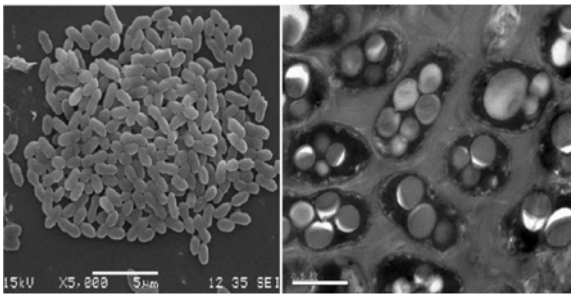

Fig.1 TEM images showing inclusion granules inside the negatively stained thin section of *Acidiphilium cryptum* DX1-1 cells (right, bar: 0.5 μm) and SEM micrograph of the cells (on left) after 40 h growth on glucose as sole carbon source (left, bar: 5 μm)

3.2 Extraction of PHB

There are no reports on extraction of PHB from *Acidiphilium* spp. In this paper, four methods were employed to extract PHB from *Acidiphilium cryptum* DX1-1 which is gram-negative strain. The results of the four methods are shown in Table 1.

Table 1 The results of four extraction methods

Extraction methods	Extraction amount/g	Extraction rate /%	Purity /%	M /(kg/mol)
SDS-NaClO	0.24	47	74	267
Lysozyme	0.19	37	89	289
Ultrasonic	0.32	62	83	213
Chloroform-sodium hypochlorite	0.37	73	92	326

Lysozyme breaks the glycosidic bonds of the polysaccharide of the bacterial cell wall. The main components of cell wall of gram-negative bacteria are lipopolysaccharides and proteins. Therefore, lysozyme has low efficiency in bacteriolysis for gram-negative strain DX1-1. On the other hand, the enzymes agglomerate in lysis solution and combine together with the macromolecules, which decrease the activity of lysozyme further. However, because of avoiding the use of toxic and explosive organic solvents, this method is used widely.

SDS acts on the lipoprotein and destroys the membrane while NaClO destroys the network structure of cell wall. They change non-PHB substance into dissoluble substance. The Anionic surfactant SDS and the oxidant NaClO complement each other in destroying the membrane. The method is attractive in industrial production, because SDS and NaClO are handy and cheap.

Ultrasonic method is effective to destroy the membrane and has high extraction rate. But the long chain of PHB is destroyed which results in small molecular weight. Viscous substance in suspension increases after treatment of ultrasonic, which makes it difficulty to extract PHB from solution.

Chloroform-sodium hypochlorite method is the best for PHB extraction of *Ac. cryptum* DX1-1: 73% extraction rate, 92% purity, with relatively slightly damage of macromolecules. After treated by sodium hypochlorite, cells break up and PHB disperse in solution. PHB were rapidly transferred to the chloroform phase, which accelerate the extraction and shorten the contact time of PHB with the sodium hypochlorite.

3.3 Characterization of the intracellular PHB

3.3.1 Ultraviolet absorption spectroscopy

When digested by sulphuric acid, PHB converted into crotonic acid, which has a maximal absorption at 242 nm. The UV spectra of sample from *Acidiphilium cryptum* DX1-1 match that of the standard completely. According to the peak value, the content of PHB can be calculated: $y=0.1629x$, y represents PHB content and x represents Abs(Fig.2). The equation serves well when Abs between 0 and 3[19]. Concentrated H_2SO_4 destroys cell membrane, and the fragments of cell have no absorption at 242 nm. Therefore, the quantity of PHB in ferment medium is calculated by digesting the cells directly.

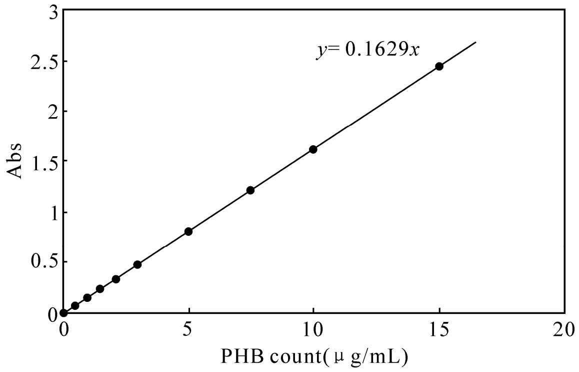

Fig.2 PHB standard curve

3.3.2 Fourier transform infrared (FT-IR) spectroscopy

Transmission electron microscope observations of negatively strain DX1-1 reveals the presence of electron-dense spherical granules (Fig.1). The FTIR spectra show the practically identical structure of the standard PHB (Fig.3a) and the biopolymers produced by *Acidiphilium cryptum* DX1-1(Fig.3b). Characteristic bands for PHB were obtained for the samples. The band found at 1457 cm^{-1} corresponds to the asymmetrical deformation of the C–H bond in CH_2 groups, while the one found at 1380 cm^{-1} is the equivalent for CH_3 groups. The bands found at 1720 cm^{-1} correspond to the stretching of the C=O bond, whereas a series of intense bands located at 1000–1300 cm^{-1} correspond to the stretching of the C–O bond of the ester group. Also bands of minor relevance, such as those found at 3440 cm^{-1}, originated by terminal OH groups or to water adsorption onto the sample, are found in all spectra. All these bands are in full agreement with those observed by Rojas de Gáscue *et al*[20]. But the OH vibration peak of the standard PHB is 92%, while that of the sample is 79%. The reason is that PHB dehydrates during the torrefaction.

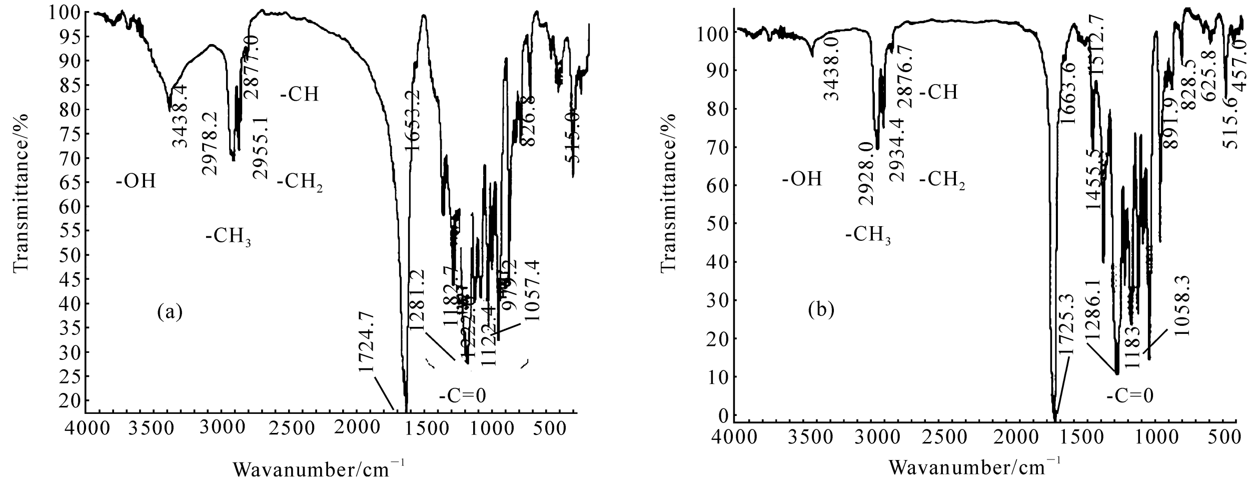

Fig.3 Fourier transform infrared spectroscopy of standard PHB (a) and PHB from strain DX1-1 (b)

3.3.3 Mass spectrometry

The mass spectrometry of standard PHB shows that the molecule weights of fragments from standard PHB are in the range of 100-2000 (Fig.4a). The molecular weight 961 which is about 9 monomers of β-hydroxybutyrate takes the higherst proportion. Fig.3b shows that the molecular weights of fragments from intracellular polymers are also in the range of 100-2000, but the molecular weight of 672 takes the highest proportion. The molecular weight of fragments from intracellular polymers is smaller than that of fragments from standard PHB. The reason is that the long chain of PHB is destroyed when treated by chloroform-sodium hypochlorite.

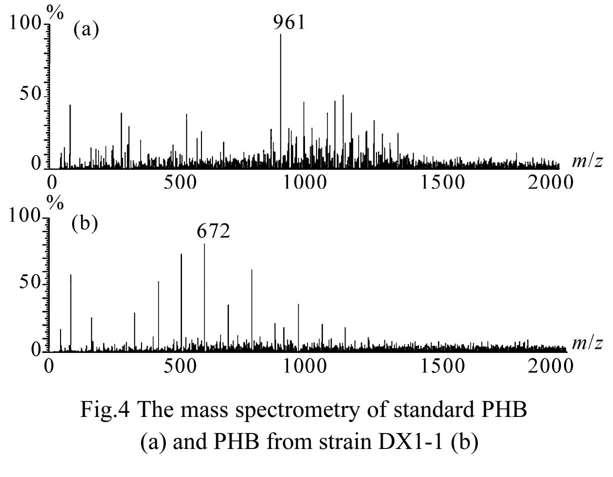

Fig.4 The mass spectrometry of standard PHB (a) and PHB from strain DX1-1 (b)

3.3.4 Nuclear magnetic resonance (NMR)

^1H NMR study was conducted to reconfirm the structure of PHB (Fig.5b). Analysis of the spectrum of PHB demonstrated that the standard contained two monomeric units. The resonance, as observed at 1.275, 2.550 and 5.256 ppm were respectively, for $–CH_3$, $–CH_2$ and OH. The ^{13}C NMR spectra are shown in Fig.5 A. The peaks observed in the spectra coincide 19.78, 40.82, 67.63, 169.15 ppm, corresponding to the different types of carbon atoms present in the PHB structure (CH_3, CH, CH_2 and C=O). If all ^1H or ^{13}C nuclei absorbed energy at exactly the same resonance frequency, NMR spectroscopy would not be very useful for determination of organic and biochemical structures. However, different ^1H or ^{13}C nuclei absorb at slightly different frequencies. This difference results from differences in the small internal magnetic field B_e for each ^1H or ^{13}C nucleus. Again, the B_e field results from the circulation of electrons of the molecule. Different ^1H or ^{13}C nuclei have different electron density surrounding them and consequently, and there are chemical shift between them. The chemical shift signals obtained in the present work is shown in Table 2, which agrees with those obtained by Doi *et al*[21] for a PHB produced by submerged fermentation. It proved that PHB from strain DX1-1 has the same biochemical structures with PHB standard.

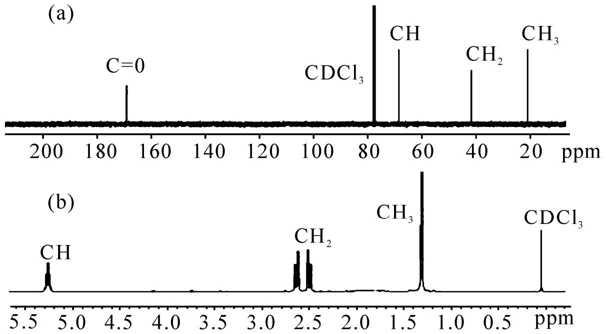

Fig.5 ^{13}CNMR (a) and ^{1}HNMR (b) spectra of PHB from strain DX1-1

Table 2 Chemical shift signals in ^{13}C NMR spectra for strain DX1-1, compared to those measured by Doi *et al*(1986) Chemical shift/ppm

C atom	Chemical shift/ppm PHB from DX1-1	Standard PHB	PHB (Doi *et al*)
CH_3	19.72	19.65	19.76
CH_2	40.76	40.66	40.77
CH	67.46	67.48	67.40
C=O	169.15	169.03	169.14

The average uncertainty for these RMN analyses is ±0.05 ppm

3.3.5 Thermal properties

The thermal behavior of the standard PHB and the samples PHB were investigated by thermal gravity and differential scanning calorimetry (DSC) analyses, and the results are shown in Fig.9. It shows the melting temperature of the biopolymer is 168 ℃, which is 9 ℃ below than the standard. The thermal properties determined for the samples and for the standard PHB are shown in Table 3. From the DSC analysis data, the melting enthalpy allows calculating the degree of crystallinity (X_c), which is the single most important characteristic of a polymer since it determines the mechanical properties of the material[22]. Highly crystalline polymers are usually brittle and find a narrower range of applications. Therefore, it is interesting to note that X_c for the standard PHB (55%), produced by submerged fermentation, is about 1.19-fold higher than *Xc* for the present samples (46%). An even higher X_c (60%) was observed by Rojas de Gáscue *et al*[20] for a PHB produced by submerged fermentation.

Table 3 Thermal properties derived from the thermogram analysis

PHB	T_m/℃	ΔH_m/(J · g^{-1})	X_c/%
Standard	177	80	55
DX1-1	168	67	46

T_m: melting temperature, ΔH_m: melting enthalpy of the sample, X_c: degree of crystallinity

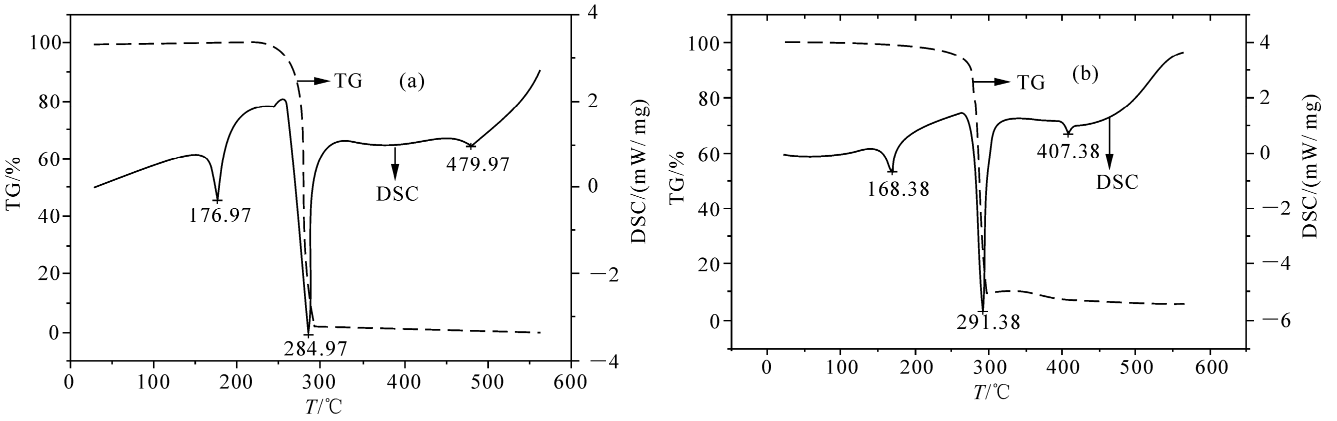

Fig.6 Thermal properties determined (a: the standard PHB and b:Extraction from DX1-1)

4 Conclusions

The best extraction method of PHB from strain *Acidiphilium cryptum* DX1-1 is chloroform-sodium hypochlorite method. This method obtained high purity of PHB, high extraction rate, relatively complete molecular structure with high molecular weight than other methods, and that is in line with the requirements of production and application.

The intracellular polymers obtained were determined by UV-visible absorption spectroscopy, Fourier transform infrared (FT-IR) spectroscopy, mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC). The results show that PHB from strain DX1-1 have the same biochemical structure and character with PHB standard. The differential scanning calorimetry (DSC) of PHB shows PHB from strain *Acidiphilium cryptum* DX1-1 has low degree of crystallinity.

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